AD_____

Award Number: W81XWH-12-1-0516

TITLE: Cadherin-11 Regulation of Fibrosis through Modulation of Epithelial-to-Mesenchymal Transition: Implications for Pulmonary Fibrosis in Scleroderma

PRINCIPAL INVESTIGATOR: Sandeep K. Agarwal, M.D., Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
 Houston, TX 77030-3411

REPORT DATE: October 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2013	Annual	30 September 2012–29 September 2013

4. TITLE AND SUBTITLE
Cadherin-11 Regulation of Fibrosis through Modulation of

Epithelial-to-Mesenchymal Transition: Imlications for Pulmonary
Fibrosis in Scleroderma

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0516

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Sandeep K. Agarwal, MD, PhD

E-Mail: Sandeep.Agarwal@bcm.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
One Baylor Plaza, BCM 310
Houston, TX 77030-3411

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Lung fibrosis is the leading cause of death in scleroderma. Treatment options are limited, stressing the unmet need to advance of understanding of the. We have demonstrated that cadherin-11 (Cad11) is increased in fibrotic skin and lung tissues and that Cad11 is a mediator of fibrosis in mouse models. Mechanistically how this occurs is not known, but our preliminary data point to a role for Cad11 in the regulation of epithelial to mesenchymal transition. In year 1 of the grant, we have performed experiments in the intraperitoneal model of pulmonary fibrosis in wild type and Cad11 deficient mice that show that Cad11 deficient mice have less lung fibrosis. Initial studies studies also suggest that antiCad11 antibodies are effective in treating lung fibrosis in this model. In vitro studies have demonstrated that Cad11 regulates epithelial-to-mesenchymal-transition (EMT) in MLE-12 cells, a mouse alveolar epithelial cell line. These data are confirming our original hypothesis that Cad11 regulates lung fibrosis through modulation of EMT. Finally, in year 1, we identified the patient sera that will be used in year 2 to determine soluble circulating levels of Cad11.

15. SUBJECT TERMS
Cadherin-11, systemic sclerosis, fibrosis, interstitial lung diseases

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	9	**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U			

Table of Contents

Progress Report Summary (October 2013, Year 1).
Grant DoD Award W81XWH-12-1-0516, Cadherin-11 Regulation of Fibrosis through Modulation of Epithelial-to-Mesenchymal Transition: Implications for Pulmonary Fibrosis in Scleroderma

INTRODUCTION.
My laboratory focuses on the potential role of cadherin-11 (Cad11) in fibrosis. We have previously reported that Cad11 expression is increased in fibrotic tissues from lungs of patients with idiopathic pulmonary fibrosis and skin of patients with systemic sclerosis. Subsequent studies have demonstrated that Cad11 is a critical mediator of lung and skin fibrosis using the intratracheal (IT) and subcutaneous bleomycin models. Preliminary studies suggest that Cad11 may regulate type 2 alveolar epithelial cell epithelial-to-mesenchymal transition (EMT), a process that contributes to the development of lung fibrosis. As opposed to the IT bleomycin lung fibrosis model, repeated administration of bleomycin via the intraperitoneal (IP) route is considered to better mimic human lung fibrosis and the process of EMT. This proposal builds on these recent observations and utilizes the IP bleomycin pulmonary fibrosis model. We hypothesize that Cad11 regulates the EMT in AEC during the development of pulmonary fibrosis and that cadherin-11 is therapeutic target in the intraperitoneal bleomycin model of pulmonary fibrosis. This proposal will be the first identify novel mechanisms by which Cad11 regulates EMT and build the foundation for additional translational studies seeking to develop Cad11 as a therapeutic target for SSc-ILD.

BODY
RESEARCH RESULTS
Specific Aim 1. Determine the role of cadherin-11 in the intraperitoneal bleomycin model of pulmonary fibrosis and the extent to which cadherin-11 modulates epithelial-to-mesenchymal transition in vivo.

In the first year of the proposal, we have performed the intraperitoneal pulmonary fibrosis model in wild type and cadherin-11 deficient mice. As seen in figure 1, wild type (WT) mice develop subpleural fibrosis when administered bleomycin (BLM). This is evidence on both H&E staining and Masson'r trichrome staining which stains the extracellular matrix and fibrosis blue. In contrast, the cadherin-11 deficient mice (KO) have an attenuated fibrotic response when repeatedly given BLM via the IP route.
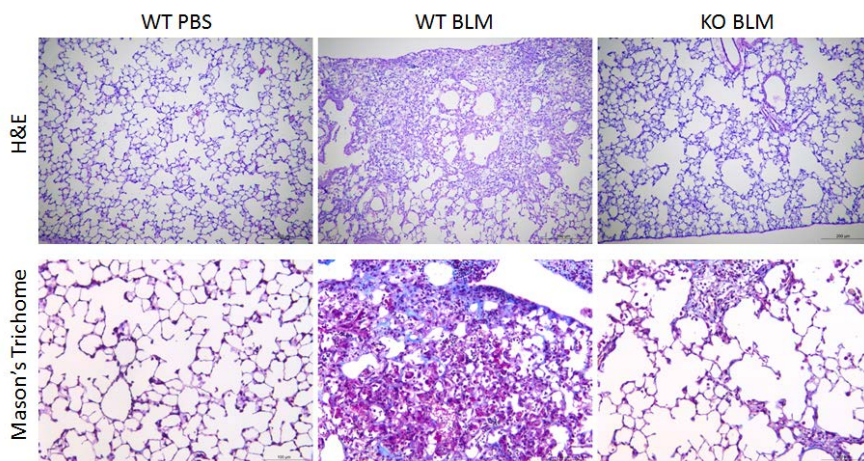

Figure 1. Pulmonary phenotype following intraperitoneal bleomycin exposure in Cad11 deficient mice. This figure displays representative histology of lungs from wild type (WT) mice treated with PBS (left) and bleomycin (BLM, middle). Cad11 deficient mice (KO) exposed to BLM are shown in the right panels. Examination of lung histology through H&E staining (top) and Mason's Trichome (MT, bottom) revealed that KO mice exposed to bleomycin displayed a reduction in lung inflammation and pulmonary fibrosis. Scale bars: 200 mm (H&E), 100 mm (MT); n= 8 mice per group

The extent of fibrosis was further quantified as seen in figure 2. WT mice had lower oxygen levels when administrated BLM but the KO mice had normal levels of oxygen, indicating less pulmonary fibrosis (fig 2a). KO mice given BLM also had lower cell counts in the bronchoalveolar lavage fluid (BAL), less BAL collagen

and lower histologic scores of fibrosis (Ashcroft Scores) compared to WT mice given BLM (figure 2). Together these data demonstrate that Cad11 deficient mice have decrease fibrosis in the IP BLM pulmonary fibrosis model and that Cad11 is an important mediator of pulmonary fibrosis.
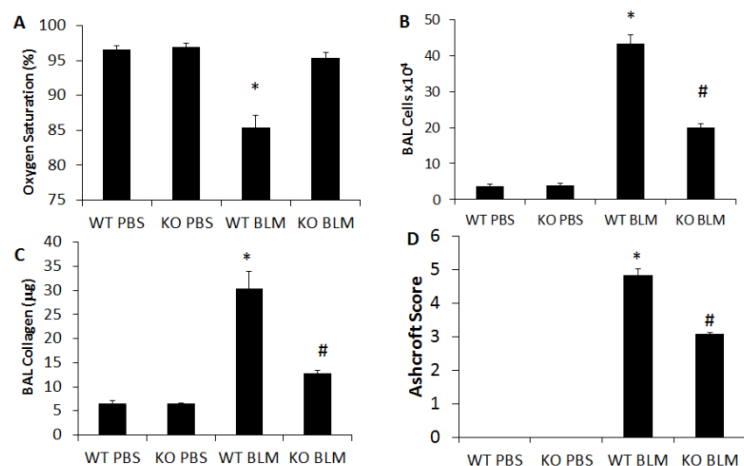

Fig 2. Quantitative analyses of fibrosis in WT and KO mice. (A) Measurement of arterial oxygen saturation. Data presented as percentage of oxygen saturation. **(B)** Total cells numbers obtained from bronchoalveolar lavage (BAL). **(C)** Soluble collagen protein levels were measured using Sircol Assay. Data presented as mean mg collagen/ml BAL fluid. **(D)** Ashcroft scores were used to determine the degree of fibrosis. Data are presented as mean ± SEM. (*, $p \leq 0.05$ WT PBS vs. WT BLM and #, $p \leq 0.05$ WT BLM vs. KO BLM; n=8 mice per group).

Preliminary studies have been conducted using the antiCad11 monoclonal antibodies as a therapeutic agent in wild type mice given IP BLM. In these experiments, treatment with isotype control or antiCad11 antibodies were started in the 3rd week of the 4 week model. As seen in figure 3, antiCad11 antibody treatment reduvced lung fibrosis as assessed by normalization of oxygen levels and lower BAL cell counts. Additional histological and quantitative analyses are being conducted at this time to confirm the role of Cad11in lung fibrosis in vivo and its potential as a therapeutic target.
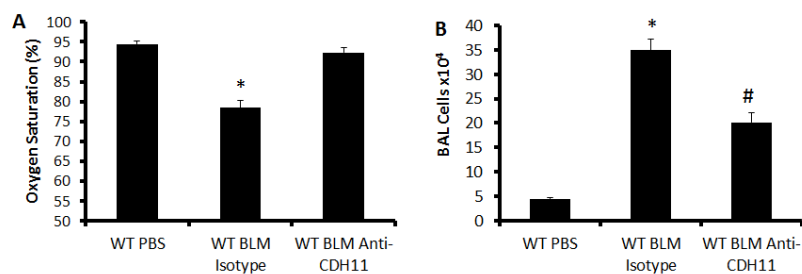

Fig 3. Treatment of pulmonary fibrosis with antiCad11 monoclonal antibodies. (A) Measurement of arterial oxygen saturation. Data presented as percentage of oxygen saturation. **(B)** Total cells numbers obtained from bronchoalveolar lavage (BAL). (Data are presented as mean ± SEM. (*, $p \leq 0.05$ WT PBS vs. WT BLM Isotype and #, $p \leq 0.05$ WT BLM Isotype vs. WT BLM Anti-Cad11; n=8 mice per group).

Finally, in year 1, we have acquired the SP-C-Cre, Rosa26 lacZ reporter mice and the breeding colony has been started for experiments in years 2 and 3. In year 2, we will complete the analyses of fibrosis in the wild type vs cad11 deficient mice as well as the antiCad11 treated wild type mice. These analyses will include additional histological analyses and immunohistological (IHC) assessment of fibrotic mediators such as alpha smooth muscle actin and beta catenin. In year 3, we will determine the expression of EMT markers in these tissues by qRTPCR and IHC.

Specific Aim 2. Determine the contribution of cadherin-11 to process of epithelial-to-mesenchymal transition (EMT) and modulation of Rho-GTPases in airway epithelial cells (AECs) in vitro.

In year 1, we have performed some initial experiments in alveolar epithelial cell lines (AEC) to determine the role of Cad11 in AEC EMT. MLE-12 cells, an AEC, were cultured and TGFbeta was used to drive the process of EMT. During EMT, E-cadherin decreases and collagen increases, therefore these mRNA transcripts were used for quantifying EMT. The results of these experiments are provided in figure 4.   To block Cad11 function, soluble Cad11 Fc fusion protein was added to cultures. As seen in figure 4A, TGFbeta decreased E-cadherin expression and increase Col1a1 expression in MLE12 cells. Soluble Cad11 Fc fusion protein inhibited EMT induced by TGFbeta as noted my higher E-cadherin levels and a significant reduction in Col1a1 mRNA. In contrast, when Cad11 Fc fusion protein was immobilized onto the tissue culture plate, providing an activating signal through Cad11 (figure 4B), a different result was observed. First, immobilized Cad11 Fc fusion protein alone was able to induce Col1a1 expression at the 50 ug/ml concentration, although E-cadherin expression was also increased. In the presence of TGFbeta, immobilized Cad11 synergistically increased Col1a1 expression, indicating that Cad11 engagement can increase collagen expression. Finally, Cad11 siRNA was utilized to block Cad11 expression in MLE12 cells (figure 4C). Cad11 siRNA alone resulted in a slight increase in E-cadherin expression and decrease in collagen expression. In the presence of TGFbeta this response was magnified where MLE12 cells transfected with Cad11 siRNA but not Ncad siRNA were unable to undergo EMT as indicated by high levels of E-cadherin and lower levels of Col1a1. These data together indicate that Cadherin-11 is a regulator of MLE12 EMT. In years 2 and 3, we will perform additional analyses of EMT in these cells as well as utilize primary AEC from wild type and Cad11 deficient mice to further determine the role of Cad11 in EMT.
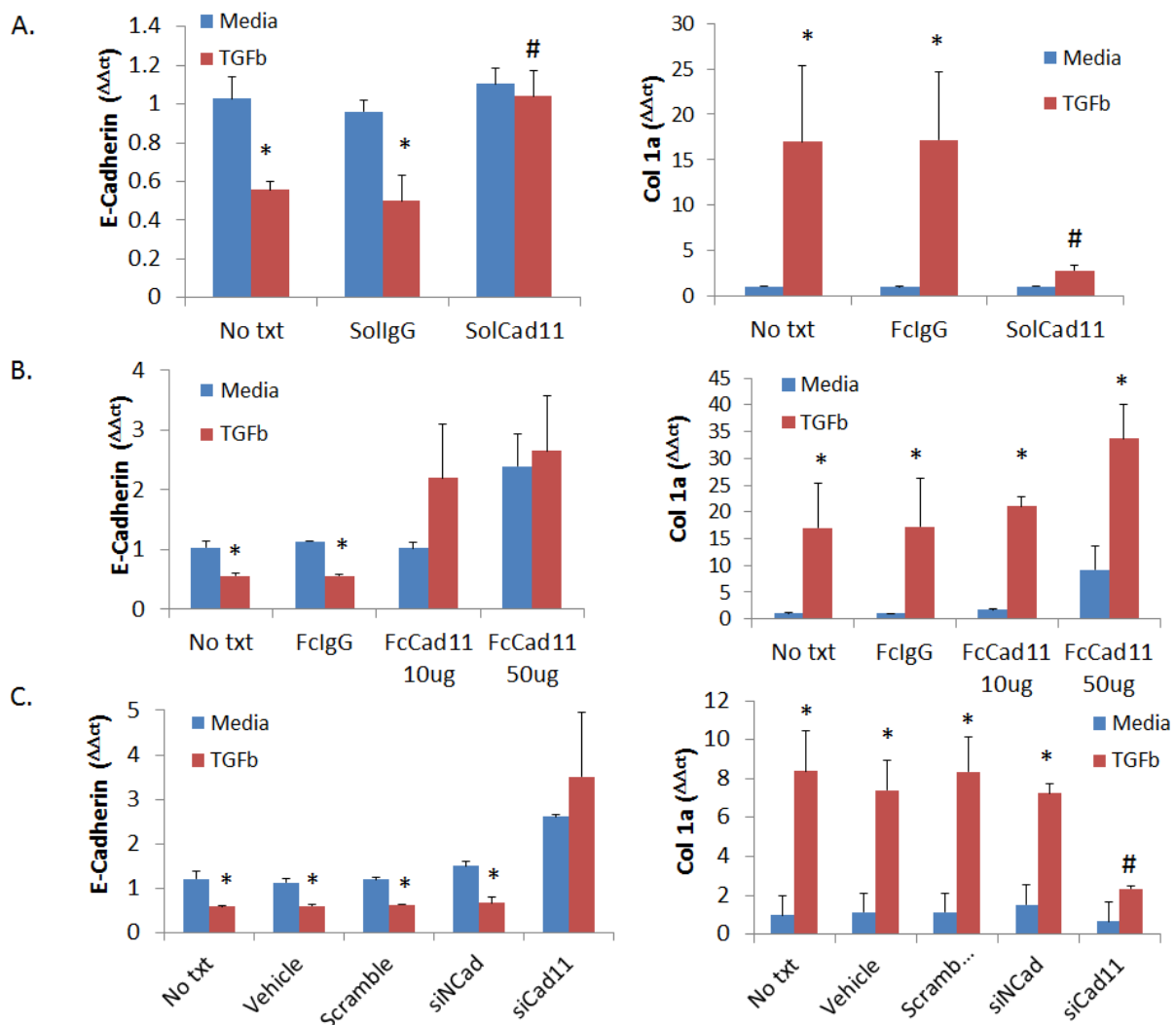
Figure 4. Cadherin-11 regulates EMT in vitro in MLE12 cells.

Specific Aim 3. Determine the circulating levels of cadherin-11 in scleroderma patients with interstitial lung disease.

We have been working with our collaborators at UTHSC to identify and sera that will be used for our soluble Cad-11 ELISA. The SSc samples have been identified and aliquotted for our ELISA detection. These include 299 patients (83% female, avg age 49, avg disease duration 2.5 years, 59% with diffuse SSc, 28% with ILD and avg skin score of 16 at enrollment). Since there is not a commercial Cad-11 ELISA, we have continued to optimize our conditions using 2 anti-Cad-11 antibodies (clones 3H10 and 23C6). As seen in figure 5, our ELISA can detect both human and mouse soluble Cad-11. In year 2 and 3, we will determine the circulating levels of Cad-11 in scleroderma patients and controls as proposed in the original Aim 3.
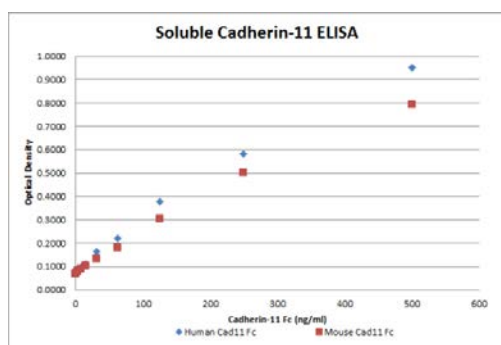
Figure 5. Soluble Cad-11 ELISA detects human (blue) and mouse (red) Cad-11

KEY RESEARCH ACCOMPLISHMENTS

1. Cadherin-11 deficient mice have decrease pulmonary fibrosis in the intraperitoneal model of pulmonary fibrosis
2. AntiCad11 antibodies are effective in treating lung fibrosis in the intraperitoneal model of pulmonary fibrosis this model
3. Cad11 regulates the in vitro TGF-beta induced epithelial-to-mesenchymal-transition (EMT) in MLE-12 cells, a mouse alveolar epithelial cell line.
4. Systemic sclerosis patient sera have been identified and aliquotted to be used in year 2 to determine soluble circulating levels of Cad11.

REPORTABLE OUTCOMES.

1. Abstract and Poster Presentation at the 2013 American College of Rheumatology Annual Meeting in San Diego, CA.
   Ref : "Pedroza M, George AT, Agarwal SK. Cadherin-11 regulates pulmonary fibrosis in bleomycin lung injury. Arthritis and Rheumatism. 65(S10):S1095, 2013."

CONCLUSIONS

Cadherin-11 is a mediator of lung fibrosis and can regulate epithelial-to-mesenchymal-transition (EMT) in MLE-12 cells, a mouse alveolar epithelial cell line.

REFERENCES
None for current report

APPENDICES
None for current report

SUPPORTING DATA
No additional data for current report, see "BODY" section above for data.